32. Lowy, P., Keighley, G. & Bonsook, H. *Nature* 185, 102–103 (1960).
33. VanLenten, L. & Ashwell, G. *J. biol. Chem.* 247, 4633–4640 (1972).
34. Lee-Huang, S. *Proc. natn. Acad. Sci. U.S.A.* 81, 2708–2712 (1984).
35. Pyrquist, F., Rosenlof, K., Gronhagen-Riska, C., Hortling, L. & Tikkanen, I. *Nature* 303, 649–652 (1984).
36. Ohkubo, H. *et al. Proc. natn. Acad. Sci. U.S.A.* 80, 2196–2200 (1983).
37. Horowitz, M., Cepko, C. & Sharp, P. A. *J. molec. appl. Genet.* 2, 147–149 (1983).
38. Bersch, N. & Golde, D. W. in *In Vitro Aspects of Erythropoiesis* (Murphy, M. J.) 252–253 (Springer, New York, 1978).
39. Cotes, P. M. & Bangham, D. R. *Nature* 191, 1065–1068 (1961).
40. Goldwasser, E. & Gross, M. *Meth. Enzym.* 37, 109–121 (1975).
41. Denman, E. *et al. Cell* 23, 731–739 (1981).
42. Anderson, S. & Kingston, I. B. *Proc. natn. Acad. Sci. U.S.A.* 80, 6836–6842 (1983).
43. Lown, R. M., Fritsch, E. F., Parker, R. C., Blake, G. & Maniatis, T. *Cell* 15, 1157–1174 (1978).
44. Woo, S. L. C. *et al. Proc. natn. Acad. Sci. U.S.A.* 75, 3688–3691 (1978).
45. Melchior, W. B. & von Hippel, P. H. *Proc. natn. Acad. Sci. U.S.A.* 70, 298–302 (1973).
46. Gross, J. M. & Wetmur, J. G. *Biopolymers* 16, 1183–1199 (1977).
47. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* 74, 5463–5467 (1977).
48. Benton, W. D. & Davis, R. W. *Science* 196, 180–182 (1977).

Identification of DNA sequences required for activity of the cauliflower mosaic virus 35S promoter

Joan T. Odell, Ferenc Nagy & Nam-Hai Chua

Laboratory of Plant Molecular Biology, The Rockefeller University, 1230 York Avenue, New York, New York 10021-6399, USA

Although promoter regions for many plant nuclear genes have been sequenced, identification of the active promoter sequence has been carried out only for the octopine synthase promoter[1]. That analysis was of callus tissue and made use of an enzyme assay. We have analysed the effects of 5′ deletions in a plant viral promoter in tobacco callus as well as in regenerated plants, including different plant tissues. We assayed the RNA transcription product which allows a more direct assessment of deletion effects. The cauliflower mosaic virus (CaMV) 35S promoter provides a model plant nuclear promoter system, as its double-strand DNA genome is transcribed by host nuclear RNA polymerase II from a CaMV minichromosome[2]. Sequences extending to −46 were sufficient for accurate transcription initiation whereas the region between −46 and −105 increased greatly the level of transcription. The 35S promoter showed no tissue-specificity of expression.

The 35S promoter region was isolated as a *Bgl*II fragment extending from −941 to +208 with respect to the transcription start site mapped for the 35S RNA found in CaMV-infected turnip leaves[3]. The polyadenylation site for the 19S and 35S CaMV transcripts located at +180 (ref. 3) was deleted, as described in Fig. 1 legend, to eliminate any possible processing signals in the promoter fragment. A 3′ deleted promoter fragment extending to +9 was deleted at its 5′ end (see Fig. 1) and fragments extending to −343, −168, −105 and −46 were chosen for analysis.

An abbreviated human growth hormone gene (*hgh*)[4] was added as a test gene downstream to the 35S promoter deletion fragments. Information on plant cell recognition of animal gene splice and 3′ polyadenylation signals obtained from analysis of *hgh* RNA transcribed in transformed plant cells will be presented elsewhere (A. Hunt, N. Chu, J.T.O., F.N. and N.-H.C., in preparation). The 35S promoter-*hgh* chimaeric gene was inserted in the pMON178 tumour-inducing (Ti)-plasmid vector, a derivative of pMON120 (ref. 5). Included in this vector is the nopaline synthase (NOS) promoter placed 5′ to the neomycin phosphotransferase-II (*npt*-II) coding region (NOS promoter-*npt*-II gene), which is co-transferred with the 35S promoter-*hgh* gene into the tobacco genome and provides an internal standard for comparison of the activities from different 35S promoter deletion fragments.

Following tri-parental matings[5,6], *Agrobacterium tumefaciens* containing both chimaeric genes was used to infect SR1 *Nicotiana tabacum* cells by wounding[5] and co-cultivation[5,7].

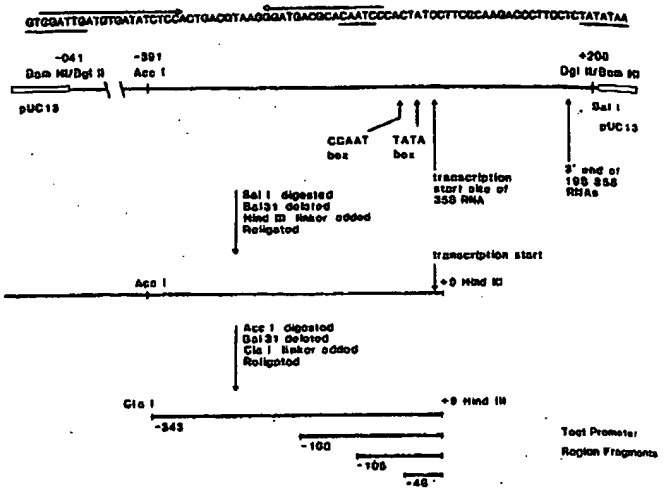

Fig. 1 Construction of 35S promoter region fragments. A 1.15-kb *Bgl*II fragment was subcloned from pCS101, a clone containing the entire Cabb-S CaMV genome[3], into the *Bam*HI site of pUC13. The resulting plasmid was linearized at the *Sal*I site in the pUC13 polylinker next to the 3′ end of the promoter fragment, digested with *Bal*31 exonuclease[11], ligated to *Hin*dIII linkers and recircularized. Clones were analysed for the extent of 3′ deletion by polyacrylamide gel sizing of the *Acc*I/*Hin*dIII fragments and finally by dideoxy sequencing[12] of subclones in pUC using the universal primer. The plasmid containing a 3′ deletion fragment with the *Hin*dIII linker at +9 was linearized with *Acc*I (site at −391), digested with *Bal*31 exonuclease, ligated to *Cla*I linkers and recircularized. Clones were analysed for the extent of 5′ deletion by polyacrylamide gel sizing of the *Cla*I/*Hin*dIII fragment, followed by dideoxy sequencing of subclones in pUC using either the universal primer or primer generation by exonuclease III digestion[13]. Above is the sequence of the −105 to −25 region of the 35S promoter[14] with TATA-box, CAAT-box, inverted repeat and core enhancer sequence regions marked.

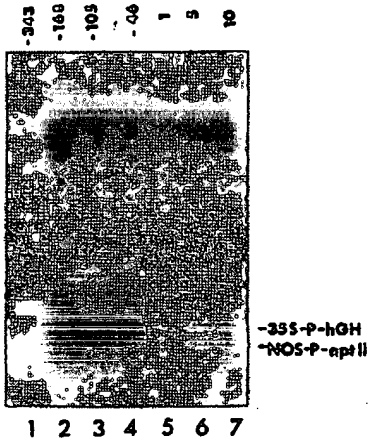

Fig. 2 Southern blot analysis of DNA from transformed tobacco calli. DNA was prepared, digested with *Eco*RI, electrophoresed on a 0.7% agarose gel and blotted onto a nitrocellulose filter[15]. A plasmid constructed to serve as the hybridization probe contains a *Bam*HI/*Sma*I *hgh* gene fragment and a *Bam*HI/*Bgl*II *npt*-II gene fragment cloned into pUC12 (GH-Neo24). The plasmid was nick translated[16] and hybridized to the Southern blot by the method of Thomashow *et al.*[17]. The following samples contain 15 μg of calli DNA transformed with: lane 1, −343 35S promoter-*hgh*; lane 2, −168 35S promoter-*hgh*; lane 3, −105, 35S promoter-*hgh*; lane 4, −46 35S promoter-*hgh*. Reconstructions of the NOS promoter-*npt*-II gene and 35S promoter-*hgh* gene copy numbers contain 15 μg of control untransformed plant DNA mixed with different amounts of the pMON178 plasmid containing the −105 35S promoter-*hgh* gene: lane 5, 17 pg = 1 copy; lane 6, 85 pg = 5 copies; lane 7, 170 pg = 10 copies. The bands near the top of the filter in lanes 1–4 result from hybridization of the pBR322 sequences in the GH-Neo24 probe plasmid to pBR322 sequences in the integrated pMON178 Ti vector. In lanes 5–7 the upper bands are derived from other regions of the pMON178 plasmid.

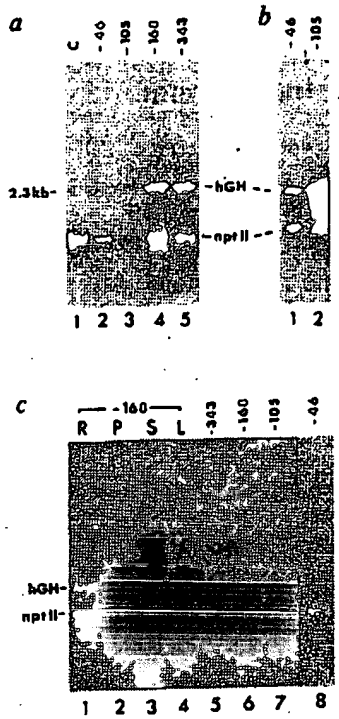

Fig. 3 Northern blot analysis of transformed tobacco calli transcripts. RNA was prepared from calli using guanidinium thiocyanate as a protein denaturant[18] and polyadenylated RNA was isolated by chromatography on poly(U)-Sepharose[19]. RNA samples were denatured with glyoxal, electrophoresed on a 1% agarose gel and transferred to nitrocellulose filters[20]. The filters were hybridized with nick-translated probes[16] for 40 h in 50% formamide, 5× SSC, 2× Denhardt's, 20 mM sodium phosphate, 0.1% SDS and 100 μg ml^{-1} denatured DNA. Filters were then washed in 2×SSC, 0.1% SDS at 23 °C followed by 0.1× SSC, 0.1% SDS at 55 °C for 1 h. *a*, Purified DNA fragments from the *npt*-II (*Bam*HI/*Hin*dIII) and *hgh* (*Bam*HI/*Sma*I) genes labelled with approximately equal specific activities (10^8 c.p.m. μg^{-1}) were hybridized to a filter containing 0.5–2 μg poly(A)$^+$ RNA from calli transformed with: lane 1, control pMON vector; lane 2, −46 35S promoter-*hgh*; lane 3, −105 35S promoter-*hgh*; lane 4, −168 35S promoter-*hgh*; lane 5, −343 35S promoter-*hgh*. *b*, Purified *npt*-II and *hgh* fragments were labelled so that the specific activity of the *npt*-II fragment was approximately 10-fold lower than that of the *hgh* fragment. Hybridization was to 2 μg poly(A)$^+$ RNA from calli transformed with: lane 1, −46 35S promoter-*hgh*; lane 2, −105 35S promoter-*hgh*. *c*, The GH-Neo24 plasmid described in Fig. 2 legend was used as the probe. Each RNA preparation was made from a pool of several plants derived from different transformation events, but carrying the same 35S promoter deletion fragment. Gel samples contain ~2 μg poly(A)$^+$RNA from: lanes 1–4, RNA from different parts of plants transformed with −168 35S promoter-*hgh*; lane 1, roots; lane 2, petals; lane 3, stems; lane 4, leaves; leaf RNA from plants transformed with: lane 5, −343 35S promoter-*hgh*; lane 6, −168 35S promoter-*hgh*; lane 7, −105 35S promoter-*hgh*; lane 8, −46 35S promoter-*hgh*.

Calli obtained by wounding were selected for kanamycin resistance (50 μg ml^{-1}) and hormone independence. Plants were regenerated from co-culture with selection for kanamycin resistance, making use of the short transfer property of the split-end vector system[8] which eliminates the T-DNA tumour genes.

Southern blots of DNA extracted from transformed calli (Fig. 2) showed the expected 1.58-kilobase (kb) *Eco*RI fragment containing the NOS promoter-*npt*-II gene and a 1.7–2.0-kb *Eco*RI fragment containing the 35S promoter-*hgh* gene, the size depending on the length of the specific promoter deletion fragment involved, indicating that no rearrangements had occurred in these two genes. Comparison with reconstructions of gene copy numbers in lanes 5–7 indicate that the copy number of these two genes varies between different transformed calli, but that there is an equal number of the two genes in each callus,

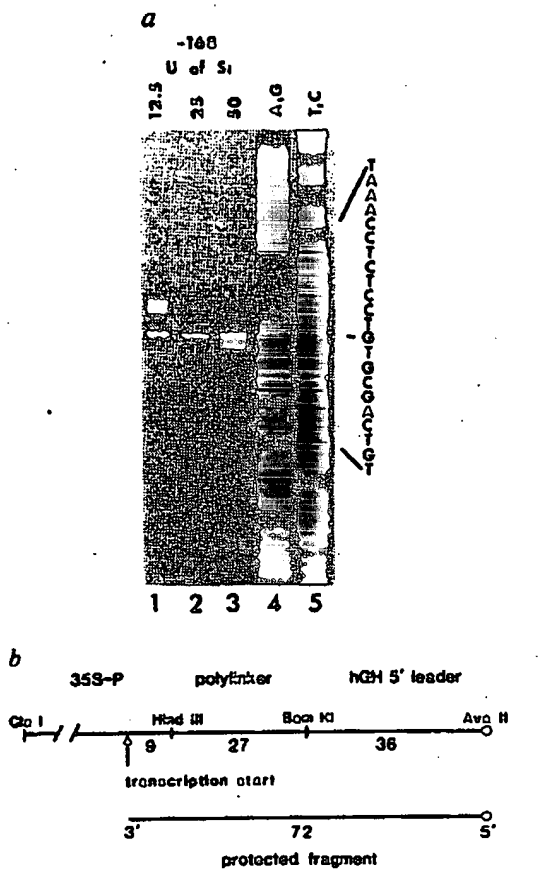

Fig. 4 *a*, S_1 nuclease mapping[21] of the 5′ end of the *hgh* transcript to the CaMV sequence. Total RNA (10 μg) from callus carrying the −168 35S promoter-*hgh* gene construction was hybridized to the probe diagrammed in *b* in 80% formamide at 37 °C. Samples were digested in 100 μl with the following amounts of S_1 nuclease: lane 1, 12.5 U; lane 2, 25 U; lane 3, 50 U. The same probe was sequenced using the Maxam and Gilbert technique[22]: lane 4, A+G reaction; lane 5, T+C reaction. All samples were electrophoresed on an 8% polyacrylamide/7 M urea gel. The DNA sequence[13] surrounding the *hgh* RNA 5′ end is shown to the right. *b*, Diagram of S_1 probe and region protected by *hgh* RNA. A *Cla*I/*Ava*II DNA fragment extending from the 5′ border of the 35S promoter fragment and into the 5′ leader region of the *hgh* transcript was 5′ end-labelled and strand separated on a polyacrylamide gel. Between the probe's labelled *Ava*II end and the 5′ end of the *hgh* RNA are 72 base pairs of S_1-protected sequence.

supporting the assumption that the two genes are co-transferred into the tobacco genome. Thus, the NOS promoter-*npt*-II gene provides a control for variables inherent in the transformation system including copy number and integration site.

Northern blots of poly(A)$^+$ RNA from transformed calli carrying each of the 35S promoter deletions showed the expected *npt*-II transcript and also a 2.3-kb transcript that contains *hgh* sequences (Fig. 3). The 35S promoter is responsible for transcription of the *hgh* RNA, as S_1 protection experiments indicate that the 5′ end of this RNA maps to the normal 35S transcription start site (Fig. 4). All the deleted promoters initiate transcription at the same site (data not shown). To compare the transcriptional activities of the different deletions, the ratio of *hgh* to *npt*-II transcripts was determined for each RNA sample. By quantitating silver grains eluted from bands on X-ray films[9], the *hgh*/*npt*-II ratios were: −46, 0.16/1; −105, 0.9/1; −168, 2.6/1; −343, 3.1/1. The *hgh*/*npt*-II ratio is the same for different RNA preparations from calli transformed with the same deletion fragment (data not shown). The −46 promoter fragment has an approximately 20-fold lower transcription level than the −343 fragment, whereas the −105 fragment has a 3-fold decrease and the −168 fragment has no significant decrease in activity. We also found

that the −343 fragment and a −941 fragment have no significant difference in promoter activity (data not shown).

Regenerated transformed tobacco plants contain one to two copies per genome of the NOS promoter-*npt*-II and 35S promoter-*hgh* genes, determined by Southern blots (data not shown). This low copy number in plants, compared with the high copy numbers in calli (Fig. 2), could be explained by the transformation procedure (co-cultivation versus wounding) or by the type of T-DNA transfer event involved (short verus long). Northern blots show that leaves of plants transformed with each of the four 35S promoter deletions contain the same 2.3-kb *hgh* RNA found in transformed calli (Fig. 3*c*). By S_1 analysis the 5′ end of this leaf *hgh* RNA was found to be identical to that of the callus *hgh* RNA (data not shown). The effects of the deletions on promoter activity in transformed plant leaves (Fig. 3*c*) closely resemble results described for the transformed calli. The 35S promoter was also active in roots, petals and stems of transformed plants (Fig. 3*c*), with deletions having no specific effects on tissue expression (data not shown). The ratio of *hgh*/*npt*-II transcripts is constant in the different tissues. Both transcripts appear reduced in the root RNA preparation, but this could be due to varying amounts of ribosomal RNA contamination in the polyadenylated RNA preparations.

Here, we have shown that although the normal host range of CaMV is limited to members of the Cruciferae, the 35S promoter is active when isolated as a fragment from the viral genome and integrated into the tobacco genome. Thus, accurate transcription from the 35S promoter does not depend on any *trans*-acting viral gene products. The ability to regenerate tobacco plants from transformed protoplasts has allowed us to demonstrate that the 35S promoter is expressed in leaves, stems, roots and petals.

Promoter deletion analysis in transformed calli and plants showed that a −46 fragment, which does contain a TATA-box sequence (see Fig. 1), produces a low level of correctly initiated transcripts. The region between −46 and −105 which greatly increases the level of transcription contains a CAAT-box sequence, an inverted repeat region and a sequence resembling the consensus core for enhancers in animal systems ($GTGG^{AAA}_{TTT}G$) (ref. 10; see Fig. 1). We are investigating whether one or more of these features plays a substantial role in increasing the level of 35S promoter activity or could act to increase transcription from a foreign promoter.

We thank Dr Ken Richards for the CaMV clone, Dr Steve Rogers for pMON178 and Dr George Pavlakis for the abbreviated *hgh* gene. We also thank I. Roberson and K. Thurman for technical assistance and Dr M. Boutry and Dr G. Morelli for helpful discussions. This work was supported by a grant from the Monsanto Company. J.T.O. holds an NIH postdoctoral fellowship (5F32AI06342).

Received 3 September; accepted 4 December 1984.

1. Konez, C. *et al. EMBO J.* 2, 1597–1603 (1983).
2. Oszewski, G., Hagen, G. & Guilfoyle, T. J. *Cell* 29, 395–402 (1982).
3. Guilley, H., Dudley, R. K., Jonard, G., Balazs, E. & Richards, K. E. *Cell* 30, 763–773 (1982).
4. Pavlakis, G. N. & Hamer, D. *Rec. Prog. Horm. Res.* 39, 353–385 (1983).
5. Fraley, R. T. *et al. Proc. natn. Acad. Sci. U.S.A.* 80, 4803–4807 (1983).
6. Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. *Proc. natn. Acad. Sci. U.S.A.* 77, 7347–7351 (1980).
7. Marton, J., Wullems, G. S., Molendijk, L. & Schilperoort, R. A. *Nature* 277, 129–131 (1979).
8. Horsch, R. B. *et al. Science* 223, 496–498 (1984).
9. Sciaky, M. *Analyt. Biochem.* 133, 511–514 (1983).
10. Glutzman, Y. & Shenk, T. (eds) *Current Communications in Molecular Biology* (Cold Spring Harbor, New York, 1983).
11. Sakonju, S., Bogenhagen, D. F. & Brown, D. D. *Cell* 19, 13–26 (1980).
12. Sanger, F., Nicklen, S. & Coulson, A. R. *Proc. natn. Acad. Sci. U.S.A.* 74, 5463–5467 (1977).
13. Guo, L.-H. & Wu, R. *Meth. Enzym.* 100, 60–96 (1983).
14. Franck, A., Guilley, H., Jonard, G., Richards, K. & Hirth, L. *Cell* 21, 285–294 (1980).
15. Southern, E. M. *J. molec. Biol.* 98, 503–517 (1975).
16. Rigby, P. W. J., Dieckmann, M., Rhodes, C. & Berg, P. *J. molec. Biol.* 133, 237–251 (1977).
17. Thomashow, M. F., Nutter, R. C., Montoya, A. L., Gordon, M. P. & Nester, E. W. *Cell* 19, 1729–1739 (1980).
18. Olsen, V., Crkvenjakov, R. & Byus, C. *Biochemistry* 13, 2633–2637 (1974).
19. Broglie, R., Bellemare, G., Bartlett, S. G., Chua, N.-H. & Cashmore, A. R. *Proc. natn. Acad. Sci. U.S.A.* 78, 7304–7308 (1981).
20. Thomas, P. *Proc. natn. Acad. Sci. U.S.A.* 77, 5201–5205 (1980).
21. Berk, A. J. & Sharp, P. A. *Cell* 12, 721–732 (1977).
22. Maxam, A. M. & Gilbert, W. *Proc. natn. Acad. Sci. U.S.A.* 74, 560–564 (1977).

Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells

John L. Yates, Noreen Warren & Bill Sugden

McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706, USA

Epstein–Barr virus (EBV) infects human B lymphocytes, transforming the infected cells into dividing blasts that can proliferate indefinitely (see ref. 1 for a review). The viral genome of 172 kilobase pairs (kbp) is a plasmid in most transformed cells[2–4]. We have identified a region of EBV DNA, termed *oriP* (nucleotides 7,333–9,109 of strain B95–8), which acts in *cis* to permit linked DNAs to replicate as plasmids in cells containing EBV DNA[5]. We have postulated the existence of a *trans*-acting gene allowing *oriP* function. Here we report that this gene lies in a 2.6-kbp region of the viral genome (nucleotides 107, 567–110, 176) which encodes the EBNA-1 antigen[6–9]. We show that circular DNAs containing *oriP*, the EBNA-1 gene and a selectable marker replicate autonomously in cells derived from at least four developmental lineages and from at least three species. We also find that the one-third of the *EBNA-1* gene repetitive in sequence is not essential for the *trans*-acting function that EBNA-1 gives *oriP*.

To map the gene encoding the proposed *trans*-acting function, overlapping segments of the EBV genome were first introduced individually into the human thymidine kinase (TK)-negative cell line 143, using a set of recombinant plasmids selected using the antibiotic G418 (ref. 5). These G418-resistant cell lines were transfected subsequently with the hypoxanthine-aminopterin thymidine (HAT)-selectable plasmid pΔπTK or its derivative, pTKBamC, which contains *oriP*. Only those G418-resistant cells that carried EBV DNA mapping from *Bam*HI-Z to *Sal*I-F (Fig. 1*a*, *b*) supported pTKBamC as a plasmid. Three of four 143 clones carrying pBamZRSalF (termed 143/BamZRSalF) could be transfected stably 5–30-fold more efficiently with the *oriP*-containing plasmid, pTKBamC, than with its parent, pΔπTK (Table 1, experiment 1). Analysis of DNA from the HAT-resistant clones showed that the 143/BamZRSalF cell lines efficiently transfected by pTKBamC contained it as a plasmid at two to four copies per cell (Table 1). 143 clones carrying all other regions of the EBV genome showed no increase in transfection frequency-dependent on *oriP* and did not contain pTKBamC as a plasmid. These results imply that the EBV DNA spanning the *Bam*HI-Z/*Sal*I-F fragment encodes the proposed *trans*-acting product which allows maintenance of *oriP*-bearing plasmids.

The only viral product encoded in this region of the genome and known to be expressed in EBV-transformed cells is the nuclear antigen EBNA-1. It is encoded in the *Bam*HI-K fragment, which lies within the *Sal*I-F fragment of EBV DNA[6–8]. A 2.9-kbp *Bam*HI/*Hin*dIII subfragment of *Bam*HI-K encodes most, if not all, of the EBNA-1 polypeptide[9,10] (see maps of Fig. 1). This 2.9-kbp subfragment was cloned into a G418-selectable plasmid containing the transcriptional enhancer of simian virus 40 (SV40), and the resulting plasmid, pSVoB-H2.9, was introduced into 143 cells. Two of five such cell lines, 143/SVoB-H2.9 clones 1 and 4, expressed levels of EBNA-1 that could be detected by anti-complement immunofluorescence (our unpublished observations). When we transfected pTKBamC into these two 143-derived clones, we efficiently selected HAT-resistant colonies in which one to three copies of the *oriP*-bearing plasmid were maintained per cell (Table 1). Thus, the *trans*-acting product required for *oriP* function maps in the 2.9-kbp region encoding EBNA-1. The SV40 enhancer was required for efficient expression of the *trans*-acting function from the integrated 2.9-kbp fragment (data not shown). This finding is consistent with the observation that the mRNA for this region is a 3.7-kb transcript beginning upstream of *Bam*HI-K[11,12].